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(54) Name of the invention: method for producing phospholipids through enzymes

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Detailed description

1. Name of the invention

method for producing phospholipids through enzymes

2. The patent's claims

(1) Method for producing phospholipids through enzymes with the following characteristics: carry out a reaction by putting a raw material phospholipid and a receptor containing a hydroxyl in contact with phospholipase D adsorbed on a vector during production of phospholipids the structure of the base of which has been transformed.

(2) Aforesaid production method of patent claim no. 1: the reaction is carried out in the presence of an organic solvent.

(3) Aforesaid production method of patent claim no. 1: the vector that adsorbed the raw material phospholipid is made to react with the receptor and vector that adsorbed phospholipase D.

(4) Aforesaid production method of patent claim no. 1: the vector is activated charcoal, activated clay, silicic acid, silica gel, diatomaceous earth, zeolite, alumina, porous glass, ceramic, porcelain or resin.

(5) Aforesaid production method of patent claims no. 1, 2, 3 or 4: the receptor is serine, ethanolamine, 1-amino-2-propanol, 1-orthomethylglucoside or trehalose.

(6) Aforesaid production method of any one of the 5 claims listed: the reaction is made with ≤1% of water contained in the reaction system.

3. Detailed explanation of the invention (Fields of industrial application)

This invention regards the method for producing phospholipids through enzymes; in particular, the method for producing phospholipids the structure of the base of which has been transformed.

(Previous technology)

Not only can phospholipids be used as emulsifiers, but over recent years also their application as a substratum of the ribosome in artificial cells, in artificial blood, in vectors of drugs and so on has been noted. Moreover, as they are endowed with their own physiological activity and a pharmacological action, many uses of phospholipids are possible in the fields of medicine, pharmacology and engineering. In order to meet these various requirements, it is extremely important that methods for effectively producing phospholipids provided with a structure corresponding to each one of their applications be developed on an industrial level.

As a method for producing phospholipids through enzymes, the technique for producing phospholipids with the desired base by making phospholipase D in the phospholipids act in the presence of any receptor and using transphosphatidilation is public property. [S.F. Yang, et al., J. Biol.Chem., 242, (3) 477-484 (1967)] : [R.M.C. Dawson, Biochem. J., 102, 205-210 (1967)].

If one plans to exchange the basic part of the phospholipids using transphosphatidilation with phospholipase D, usually the two-stage system of the aqueous stage and of the organic solvent is used. In other words, it is a reaction system in which an organic solution, above all containing raw material phospholipids that are lipophilic, is mixed and agitated with an aqueous solution that above all contains water-soluble enzymes, inorganic salts, the pH buffer solution, the receptor and so on. The technology cited above is widely used also in research subsequent to the one cited, such as [K. Bruzik and M. Tsai, Biochemistry 23, (8) 1656-1661 (1994)].

(Problems that the invention proposes to solve)

Nevertheless, this kind of reaction system used up until now causes a considerable quantity of water to be present as solvent for the water-soluble components, and seeing that phospholipase D basically has a hydrolytic activity, hydrolysis occurs as a secondary reaction and has the defect of forming phosphatidic acid (from now on abbreviated PA).

The formation of PA following hydrolysis does not limit itself to complicating the separation and purification of the raw material phospholipids, the object after the reaction. Essentially, it was not possible to get the desired result because the raw material phospholipids are consumed with the hydrolytic reaction as well, so if we intend to bring on the extension of the phosphatidile group as regards low-reactivity receptors such as, for example, sugar or secondary alcohol, their reaction speed is extremely low compared to the reaction speed of the hydrolysis.

Problems of this type are inevitable as long as water is present since phospholipase D is essentially a hydrolase.

So the inventors of this patent have solved this problem by reducing to a minimum the quantity of water contained in the reaction system, within the bounds in which the enzyme does not become inactivated, and, following various studies, they have found a new reaction system that adds the components that were previously added to the reaction system as aqueous solution through support or adsorption on the vector, and hence arrived at formulating this invention.

(Steps for solving the problems)

The characteristic of this invention is to carry out a reaction by putting a raw material phospholipid and a receptor containing a hydroxyl in contact with phospholipase D adsorbed in a vector.

The raw material phospholipid used in this invention can be any natural extract or substance purified after extraction or a synthesized substance as long as it is able to act as a substratum of phospholipase D. Furthermore, substances on the market or substances prepared with methods of common knowledge can be used without distinction. What can be cited as examples are scoured soy lecithin, egg yolk lecithin, phosphatidylcholine (hereinafter abbreviated PC), phosphatidylethanolamine (hereinafter abbreviated PE), phosphatidylserine (hereinafter abbreviated PS), phosphatidylglycerol (hereinafter abbreviated PG), etc., or mixtures of these substances. To get the maximum yield of the effects of this invention, use as the raw material phospholipid purified substances or substances with a simple composition as it lends itself better to purification of the reaction's product. Furthermore, from the raw material costs and ease of production point of view, as well as for the reactivity towards the enzyme it is preferable to use PC, PE or PS in particular, which give excellent results on an industrial level.

It is preferable to make the reaction in the presence of an organic solvent that dissolves or suspends the raw material phospholipid, and any substance can be used as long as it is a system of solvents that inactivates few enzymes. We can cite as examples petroleum ether, diethylether, methylethylether, diisopropylether, chloroform, dichloromethane, carbon tetrachloride, dichloroethane, n-hexane, cyclohexane, n-octane, non-octane, ethylacetate, dioxane, benzene and other solvents, or systems of solvents obtained from mixtures of the aforesaid substances or mixed systems of solvents in which polar solvents are combined, such as, for example, acetone, acetonitrile, etc. However, it is preferable to use alcohol only for adding it as a substratum so that it becomes the substratum of the object reaction.

Like phospholipase D, any substance on the market or some substance produced with methods of public property can be used as long as it is a substance with transphosphatidilation. What can be cited as examples are the phospholipase D derived from cabbage and produced by the Boehringer Mannheim GmbH company, the phospholipase D derived from micro-organisms (PLDP) and produced by the Tōyō Jōzō Ltd. company, and extracted and purified enzymes, enzymes partially purified or crude enzymes extracted with methods of public property (i.e. the method of M. Kates and P.S. Sastry "Methods in Enzimology", J.M. Lowenstein, ed., vol. 14, pp 197-203, Achademic Press, New York, 1969).

As a receiver, we can use not only the compounds known to date as receivers of the transtransfer of phosphatidyl – for example choline, methanol, ethanol, ethanolamine, serine, glycerol, glucose and so on – but we can also use compounds having a structure of primary or secondary alcohols containing saccharides and that until now were not considered receivers of the transtransfer of phosphatidyl, starting with 1-amino-2-propanol, 1-orthomethylglucoside, and trehalose.

As regards a vector that adsorbes or supports phospholipase D, on the material level we can cite active charcoal, activated clay, silicic acid, silica gel, diatomaceous earth, zeolite, alumina, porous glass, ceramic, porcelain, resin and so on. On the shape level, the granular shape with a diameter of approx. 0.02 to 0.5 mm or the pearl shape is preferable. Moreover, these vectors are also able to adsorbe and support the raw material phospholipid and the receiver.

Various methods exist as examples of methods for supporting or adsorbing phospholipase D and the receiver in the vector. There is the method in which the aqueous solution with the above-mentioned components is put into contact and is supported with the vector, and the water in excess after filtering is lyophilised or eliminated by the dry organic solvent's

repeated or interrupted contact that does not inactivate the enzyme, such as, for example, diethylether or chloroform. Or there is the method in which a minimum quantity of water is added in the point where the vector and the above-cited components broken down into powder have been mixed and are then mixed again, etc.

There are various methods for putting the organic solvent that dissolved or suspended the raw material phospholipid in contact with the vector that adsorbed or supported the enzyme, the receiver and other components. There is the method with which you disperse, suspend and agitate the vector inside the system of solvents in a container, or the method with which you fill a column with the vector and you make the system of solvents, etc. circulate.

Various methods exist as examples of methods for adsorbing or supporting the raw material phospholipid in the vector. There is the method in which the solvent is eliminated by filtration after putting in contact and making adsorb with the vector the raw material phospholipid dissolved in a low-polarity organic solvent; or the method for removing the solvent after having saturated the organic solvent that dissolved the raw material phospholipid with the vector.

The hydrolytic reaction can be kept under control compared to the previous reaction system by reducing the quantity of water contained in the entire reaction system to preferably ≤1%, but it would be even more advisable to reduce it to ≤0.5%. In particular, by reducing the water content to ≤0.2% it is possible to make the receivers that were unable to react in the previous reaction system react too, and the effects of this invention offer the maximum yield.

The reaction temperature can be the enzyme's optimum temperature of use, generally between 30°C and 40°C. However, if, for example, the solvent used has a low boiling temperature, the limit will be different.

The reaction time ranges between 0.5 and 36 hours, preferably between 4 and 24 hours.

The object phospholipid, provided with a random base produced this way, can easily be purified using instruments of public property in the proper manner, such as, for example, fractioning of the solution, chromatography on silicic acid or silica gel, chromatography on alumina, DEAE-cellulose chromatography and so on.

(Effects of the invention)

In this invention, we have essentially used a non-aqueous system to reduce the quantity of water present in the reaction system to the minimum within the limit in which the enzyme does not inactivate, so the formation of a large quantity of PA like that we found in the previous reaction system is kept under control. Separation and purification of the object phospholipid following the reaction become easy and the yield is higher.

We were also able to produce numerous types of object phospholipid, whereas this was not possible with the previous reaction system.

(Examples)

The invention is explained hereunder in a concrete manner based on examples of reference, practical examples and comparative examples.

The analysis of the composition and the examination of the phospholipid's pureness were carried out by thin-layer chromatography (TLC). We detected between 20 and 100 μg of lipid samples in a diameter of between 3 and 5 mm on a TLC plate (manufactured by the Merck Company, No. 5721) and we generated chloroform-methanol-water (120:70:5) or chloroform-acetone-acetic acid-methanol-water (50:20:15:10:5). We used the Dittmer reagent, 50% sulphuric acid, and the reagent ninhydrine or the reagent anthrone, according to the objective, for the observation. For the quantitative measurement, we measured the substance coloured with the Dittmer reagent with the thin layer chromatoscanner (model CS-920, manufactured by the Shimizu factory).

Furthermore, we identified the reaction's result – unless recorded differently – by the reaction to the colouring for each reagent and by comparison between the Rf values on TLC and the genuine sample.

Example of reference 1

We extracted spinach phospholipase D by following the method of M. Kates and P.S. Sastry, an example of the methods of public property mentioned above. After having rinsed fresh spinach (Parade variety) purchased from a farm in the neighbourhood, we cut it up, we added 200 ml of water to 100 g of spinach and we homogenized it in ice for 5 minutes. For 15 minutes we centrifuged 2,000 × g at 4°C the filtered liquid through a gauze folded over 5 times and we obtained 210 ml of supernatant. We dialysed this supernatant 3 times for 1 l of water at 4°C and we used the 195 ml of supernatant centrifuged 15 minutes 10,000 × g at 4°C as crude spinach enzyme. We verified that no phospholipids in a noticeable quantity were contained in this crude enzyme liquid.

Example of reference 2

We separated and fractionated the soy PC and the PE with the Pardun method [Von H. Pardun – Fette Seifen Anstrichmitte 86, (2) 55-62 (1984)].

We dispersed 20 g of scoured soy lecithin in powder form found on the market (24% PC, 21% PE, 14% phosphatidylinositol, 8% PA) in 100 ml of sopropanol-methanol-water (50:45:5), we heated and agitated it at 40°C and we dissolved it. We cooled it down to 20°C while continuing to agitate it, and we kept it at 20°C for 1 hour. We centrifuged and filtered the insoluble substances at low pressure with a glass filter while keeping them at 20°C. We dried the supernatant gathered at low pressure and we obtained 9.7 g of PC and PE concentrate (68% PC, 17% PE, 7% PA, not containing PS).

Example of reference 3

We purified the PC from the egg yolk lecithin with the usual method. We fractionated 10 g of egg yolk lecithin found on the market (67% PC, 19% PE, 8% PA, 3% lysoPC) with a silica gel column (diameter: 3.8 cm × 60 cm). We poured 1.5 chloroform-methanol (5:1) and 3 l of chloroform-methanol (3:1) as elution solvent, we gathered the fraction containing a large quantity of PC and, after having dried it at low pressure, we obtained 4.3 g of PC (97% PC).

Example of reference 4

We supported the purified egg yolk PC in a piece of porous glass. We dissolved 500 mg of purified egg yolk PC obtained with example of reference 3 in 100 ml of n-hexane and, after saturating it in 5 g of porous glass broken into a diameter of less than 0.5 mm, we supported it by drying the n-hexane at low pressure.

Example of reference 5

We extracted the crude cephalin from the brain of a bovine with the Lees method [M. Lees – "Methods in Enzymology" (S.P. Colowick and N.O. Kaplan ed.) vol.3, pp328, Achademic Press, New York (1957)] and we purified it with DEAE-cellulose column chromatography.

We homogenized 300 g of fresh bovine brain bought in a local slaughterhouse, from which the membrane and the blood vessels were removed, in 1.2 l of acetone and then we extracted. We extracted the filtered cake another time with 1.2 l of acetone. We extracted the filtered cake in 1.2 l of ethanol. We extracted 1.2 l of petroleum ether twice with the same procedure adopted for the filtered cake. We gathered the liquid extract and then we dried it at low pressure, in this way obtaining 3.9 g of fraction of crude cephalin.

We dissolved this substance in chloroform and we fractionated it with a DEAE-cellulose column (DE32, manufactured by the Whatman company, diameter: 2.5 × 20 cm) prepared in acetic acid. After having washed the column with 1 l of chloroform-methanol (1:4), we collected the eluted fraction with 750 mm of acetic acid. We added isovolumetric chloroform to the eluate fraction with acetic acid and we washed 4 times with the double

volume of water. We dried the layer of chloroform at low pressure and we obtained the PS (98% PS).

Example of reference 6

We washed the diatomaceous earth found on the market with water and the solvent. We removed the particles through decantation and at the same time we suspended and washed 100 g of celite No. 503 (manufactured by the Johns-Manville Sales company, registered trademark) in 2 l of water. Following the same procedure, we again washed with water 2 times, with methanol 1 time, with chloroform 1 time and in the end we filtered the suspended substance in 500 ml of acetone at low pressure. After having air-dried it, we dried it for 5 hours at 120°C and we obtained 74 g of purified celite.

Example 1

We dissolved 50 g of L-serine, 0.5 g of cabbage phospholipase D (manufactured by the Boehringer Mannheim company), 0.25 g of calcium chloride – dehydrated in 500 ml of 5 mM acetic acid buffer solution with pH 5.6, we added 40 g of superior quality activated charcoal (manufactured by the Wako Pure Chemicals Industries, Ltd. company) and we agitated for 30 minutes at room temperature. Following separation by filtration, we lyophilised for 8 hours and we obtained 53.7 g of activated charcoal that adsorbed enzymes and receivers.

We dissolved 8 g of PE concentrate and soy PC obtained with example of reference 2 in 500 ml of diethylether, we added 53.5 g of activated charcoal that had adsorbed enzymes and receivers, and we dispersed. We kept the temperature at 37°C in a hermetically sealed container and we caused the reaction by agitating the substance for 17 hours at 500 rpm.

We recovered the reaction solvent through filtration and we washed the activated charcoal 3 times with 200 ml of chloroform. We dried at low pressure, mixing the washing liquid with the reaction solvent, and we obtained 6.9 g of phospholipid mixture (8% PC, 8% PE, 9% PA, 72% PS).

We dissolved this substance in the chloroform and then we fractionated with a DEAE-cellulose column (DE32, manufactured by the Whatman company, diameter: 3.8 × 27 cm). After having washed the column with 1.5 l of chloroform-methanol (1:4), we followed the same procedure as illustrated in example of reference 5 from the eluate faction with 1.2 l of acetic acid and then we collected 3.4 g of PS (99% PS).

Example 2

We added 2.5 g of purified celite obtained through example of reference 6 to the liquid mixture made up of 25 ml of crude spinach enzyme liquid, prepared with example of reference 1, and 25 ml of aqueous solution of ethanolamine 1M adjusted to pH 5.0 with chloridic acid and we agitated it for 30 minutes at room temperature. After filtration, we suspended a small amount of diisopropylether and we filled the column (diameter: 1 × 3.5 cm). We ran 2.5 l of dry diisopropylether and then we absorbed the excess water; after having removed it, we used a metering pump to make 20 ml of diisopropylether circulate, in which we dissolved 3 g of egg yolk lecithin found on the market (67% PC, 19% PE, 8% PA, 3% PC) for 6 hours at 30°C and at a flow rate of between 0.3 and 0.5 ml/min., in this way bringing about the reaction.

We recovered the reaction solvent and we washed the column 3 times with 10 ml of chloroform. We dried at low pressure while mixing the washing liquid with the reaction solvent, and we obtained 2.7 g of phospholipid mixture (1% PC, 85% PE, 10% PA, 2% lysoPC).

We dissolved this substance in the chloroform and then we fractionated with a silica gel column (diameter: 2.5 × 40 cm). We eluted it with 2 l of chloroform-methanol-water

(65:25:2), we collected the fraction containing PE and we dried it at low pressure, thus obtaining 1.6 g of PE (97% PE).

Example 3

We dissolved 50 mg of phospholipase D (manufactured by the Boehringer Mannheim company), 0.5 g of calcium chloride – dehydrated in 2 ml of aqueous solution of 333 mM 1-amino-2 propanol adjusted to pH 5.5 with chloridic acid, we added 200 mg of purified celite obtained through example of reference 6 and we mixed well for 15 minutes. We lyophilised the precipitate for 6 hours, centrifuged for 15 minutes at 12,000 × g and we obtained 278 mg of celite that had adsorbed enzymes and receivers.

We suspended and dispersed 276 mg of celite, which had adsorbed enzymes and receivers, and 600 mg of porous glass, which had supported the purified egg yolk PC obtained through example of reference 4, in 10 ml of n-hexane-acetone (95:5). We agitated at 500 rpm for 24 hours at 37°C, thus causing the reaction.

We recovered the reaction solvent through filtration and we washed the vector 3 times with 5 ml of chloroform. We dried at low pressure while mixing the washing liquid with the reaction solvent, and we obtained 42 mg of phospholipid mixture.

This substance contains 4% PA, but the PC is not detected and since the substance representing the remaining 94% is positive both to the Dittmer reagent and to the ninhydrine reagent and the TLC's Rf values are very similar to the standard PE, we determined that it is the object phospholipid in which the phosphatidyl group was introduced into the 1-amino-2-propanol.

Example 4

We carefully mixed 100 mg of lyophilised powder of the crude spinach enzyme liquid obtained through example of reference 1 and 300 mg of activated charcoal. We added 60 mg of glycerol (percentage of water: 3%) and then we amalgamated well. We dispersed this substance in 15 ml of chloroform in which 50 mg of bovine brain PS was dissolved, obtained through example of reference 5, and we agitated at 500 rpm for 5 hours at 35°C, in this way bringing about the reaction.

We recovered the reaction solvent by filtration and we washed the activated charcoal 3 times with 5 ml of chloroform. We dried at low pressure while mixing the washing liquid with the reaction solvent, and we obtained 44 mg of phospholipid mixture (28% PS, 3% PA, 67% PG).

Example 5

We dissolved 12 µg of phospholipase D (PLDP, manufactured by the Tōyō Jōzō Ltd. company) and 600 mg of 1-orthomethylglucoside in 5 ml of 5 mM acetic acid buffer solution with pH 5.6 and, by following the same procedure described in example 3, we adsorbed in 1 g of activated charcoal and we lyophilised. We dispersed 950 mg of activated charcoal that had adsorbed enzymes and receivers in 15 ml of chloroform-isooctane (1:1) in which we dissolved 45 mg of dipalmitoilPC. We agitated at 500 rpm for 12 hours at 38°C, thus causing the reaction.

By following the same procedure as example 4, we obtained 48 mg of phospholipid mixture.

We used this substance with the TLC plate for fractionation No. 5745 (manufactured by the Merck company). As the developing solvent, we fractionated and dispersed chloroform-methanol-water (120:70:5) and we obtained 29 mg of unidentified phospholipid. When we analysed this phospholipid using the JMS-DX303 mass spectrometer (manufactured by the JEOL, Ltd. company) in the conditions given below, the peak from the cationic part was m/e 847 and the peak from the anionic part was m/e 823.

Measurement conditions

Ionisation method: FAB method

Impact gas: Xe

Primary acceleration voltage: 6 kV
Filament current: 20 mA
Detector: conversion dynode
Extrusion voltage: 15 kV
Matrix: triethanolamino (in case of cation, add sodium chloride)
Data management: JMA-DA5000
We identified the object phospholipid in these values because when the phosphatidyl group was put into the 1-orthometylglucoside, each sodium salt (molecular weight 824 + 23), of which we had assumed the molecular weight (824), coincided with the anion (molecular weight 824 –1).

Example 6

We dissolved 1 g of trehalose and 10µg of phospholipase D (PLDP, manufactured by the Tōyō Jōzō Ltd. company) in 5 ml of water. We agitated for 5 hours at 4°C while adding a styrene-divinylbenzene resin in dry pearls found on the market and we saturated. Following filtration, we dispersed the lyophilised substance for 18 hours in 20 ml of dry dichloromethane in which we dissolved 45 mg of dipalmitoilPC, and we mixed by turning at 40 rpm for 24 hours at 37°C, in this way bringing about the reaction.
We recovered the reaction solvent with filtration and we washed the resin 3 times with 20 ml of dichloromethane. We dried at low pressure while mixing the reaction solvent and the washing liquid, and we obtained 43 mg of phospholipid mixture.
This substance contained 38% PC and 5% PA, but the phospholipid that represented the 54% was positive to the Dittmer reagent and to the anthrone reagent, so we determined that it is the object phospholipid in which the phosphatidyl group is introduced in the trehalose.
Furthermore, we identified the object phospholipid by following the same procedure as described in example 5 because we partially fractionated the substance with TLC and once the mass spectrometry was done, the peak from the cationic part was m/e 995, the peak from the anionic part was m/e 971 and each sodium salt (molecular weight 972 + 23) with the calculated molecular weight (972) corresponded to the anion (molecular weight 972 – 1).

Comparative example 1

The reaction was carried out in conditions as similar as possible to those of example 1 in the foregoing reaction system. We agitated 5 ml of diethylether in which we dissolved 40 mg of PE concentrate and soy PC obtained through example of reference 2 and 5 ml of 50 mM acetic acid buffer solution with pH 5.6 containing 2.5 mg of phospholipase D (manufactured by the Boehringer Mannheim company), 200 mM L-serine and 40 mM calcium chloride at 500 rpm and at 37°C.
We had 5 identical samples react in parallel, each for 1, 2, 4, 8 and 16 hours. After the reaction we extracted the lipid with 5 ml of diethylether 3 times and we analyses the phospholipid's composition. The results are illustrated in Table No. 1.

Table No. 1

	Composition %			
Reaction time (h)	PC	PE	PA	PS
1	15	31	42	12
2	8	12	63	7
4	0	3	88	8
8	0	0	97	2
16	0	0	99	0

Comparative example 2

We carried out the reaction in conditions as similar as possible to those of example 2 with the foregoing reaction system.

We agitated 5 ml of diisopropylether in which we dissolved 45 mg of egg yolk lecithin found on the market, 4.275 ml of water, 0.375 ml of crude spinach enzyme liquid obtained through example of reference 1 and 0.35 ml of aqueous solution of 1 M ethanolamine adjusted to pH 5.0 with chloridic acid at 500 rpm and at 30°C.

We made 4 identical samples react in parallel, each for 1, 2, 4 and 6 hours, and we analysed them with the same procedure adopted for comparative example 1. The results are illustrated in Table No. 2.

Table No. 2

	Composition %			
Reaction time (h)	PC	PE	PA	LysoPC
1	18	40	36	5
2	5	33	52	8
4	3	19	73	3
6	0	8	85	4

Comparative example 3

We carried out the reaction with the foregoing reaction system in conditions as similar as possible to those of example 5.

We agitated 5 ml of chloroform-isooctane (1:1) in which we dissolved 45 mg of dipalmitoilPC and 5 ml of 50 mM acetic acid buffer solution with pH 5.6 in which we dissolved 12 μg of phospholipase D (PLDP, manufactured by the Tōyō Jōzō, Ltd. company) and 600 mg of 1-orthomethylglucoside at 500 rpm and at 38°C.

We analysed them chronologically by following the same procedure illustrated in comparative examples 1 and 2, but the spot corresponding to the object phospholipid found with example 5 was not found on the TLC and we stopped the analysis because the matrix was completely analysed in 8 hours of reaction.

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⑤4発明の名称 酵素によるリン脂質の製造方法

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明細書

1. 発明の名称

酵素によるリン脂質の製造方法

2. 特許請求の範囲

(1) 塩基構造が変換されたリン脂質を製造するにあたり、原料リン脂質と水酸基を有する受容体とを、担体に吸着させたホスホリパーゼDに接触させて反応を行うことを特徴とする酵素によるリン脂質の製造方法。

(2) 反応を有機溶媒の存在下で行う特許請求の範囲第1項記載の製造方法。

(3) 原料リン脂質を吸着させた担体と、受容体およびホスホリパーゼDを吸着させた担体とを混合し反応させる特許請求の範囲第1項記載の製造方法。

(4) 担体が活性炭、活性白土、ケイ酸、シリカゲル、ケイ藻土、ゼオライト、アルミナ、多孔質ガラス、陶器、磁器、または樹脂である特許請求の範囲第1項記載の製造方法。

(5) 受容体が、セリン、エタノールアミン、1-アミノ-2-プロパノール、1-オルソメチル-グルコシド、トレハロースのいずれかである特許請求の範囲第1、2、3または4項記載の製造方法。

(6) 反応系中の水分含量が1%以下の状態で反応を行う特許請求の範囲第1~5項のいずれか1項に記載の製造方法。

3. 発明の詳細な説明

(産業上の利用分野)

本発明は、酵素によるリン脂質の製造方法に関し、特に、塩基構造が変換されたリン脂質を製造する方法に関する。

(従来の技術)

リン脂質は、単に乳化剤に用い得るのみならずリポソームの基材として薬剤運搬体、人工血液、人工細胞等への応用が近年注目されており、また、それ自体生理活性・薬理作用を持つものとして、医学・薬学・工業的分野の様々な用途が考えられている。このような多様な要求に対応するために、各々の用途に応じた構造を有するリン脂質を効率

的に製造する方法を開発することは、産業上非常に意義あることである。

酵素によるリン脂質の製造方法として、リン脂質にホスホリパーゼDを任意の受容体の存在下に作用させ、ホスファチジル基転移反応を利用して目的とする塩基を持つリン脂質を製造する技術は公知である〔S.F.Yang, et al., J.Biol.Chem., 242,(3) 477~484 (1967)〕；〔R.M.C.Dawson, Biochem. J.,102, 205~210 (1967)〕。

ホスホリパーゼDによるホスファチジル基転移反応を利用してリン脂質の塩基部分を交換しようとする場合、一般に水相と有機溶媒相との二相系が用いられる。すなわち、主として水溶性である酵素、受容体、pH緩衝液、無機塩等を含む水溶液と、主として親油性である原料リン脂質を含む有機溶媒相とを攪拌・混合する反応系である。前出の技術をはじめ、その後の研究（K.Bruzik and M.Tsai, Biochemistry 23, (8) 1656-1661(1984) など）においても広く用いられている。

（発明が解決しようとする問題点）

しかし、従来用いられていたこのような反応系は、水溶性成分の溶媒としての多量の水の存在が原因となり、ホスホリパーゼDが本質的に加水分解活性を持っているために、副反応として加水分解が起こり、ホスファチジン酸（以下PAと略す）を生成するという欠点を有している。

加水分解によるPAの生成は、反応後の目的リン脂質の分離精製を困難にするばかりでなく、加水分解反応によっても原料リン脂質が消費されるため、糖や二級アルコール等の反応性の低い受容体に対してホスファチジル基を転移させようとする場合、その反応速度が加水分解の反応速度に対して極端に低いために事実上目的生成物を得ることができなかった。

このような問題点は、ホスホリパーゼD自体が本来加水分解酵素である以上、水が存在する限り不可避である。

そこで、本発明者らは、反応系中の水分含量を酵素が失活しない範囲で極限まで減少させることによりこの問題を解決すべく、種々検討の結果、従来水溶液として反応系に添加していた成分を、担体に吸着あるいは担持せしめて添加する新規な反応系を見出し、本発明に至ったものである。

（問題点を解決するための手段）

本発明は、原料リン脂質と、水酸基を有する受容体とを、担体に吸着させたホスホリパーゼDに接触させて反応を行うことを特徴とする。

本発明において用いられる原料リン脂質としては、ホスホリパーゼDの基質となり得るものであれば、天然から抽出したもの、または抽出後精製したもの、あるいは合成したものを問わず使用できる。また、市販のものあるいは公知の方法で調製したものを使用しても差し支えない。

例として脱脂大豆レシチン、卵黄レシチン、ホスファチジルコリン（以下PCと略す）、ホスファチジルエタノールアミン（以下PEと略す）、ホスファチジルセリン（以下PSと略す）、ホスファチジルグリセロール（以下PGと略す）等またはそれらの混合物等があげられる。本発明の効果を最大に発揮するためには、原料リン脂質として精製したものないしは組成の単純なものを用いた方が反応生成物の精製の面で都合が良い。また、原料コストと入手の容易さ、酵素に対する反応性の面から特にPC、PEまたはPSが工業的に効果が高く好ましい。

反応は、原料リン脂質を溶解または懸濁する有機溶媒の存在下で行うことが好ましく、酵素を失活させることの少ない溶媒系であればいずれも使用できる。例として、石油エーテル、ジエチルエーテル、メチルエチルエーテル、ジイソプロピルエーテル、クロロホルム、ジクロロメタン、四塩化炭素、ジクロロエタン、n-ヘキサン、シクロヘキサン、n-オクタン、イソオクタン、酢酸エチル、ジオキサン、ベンゼン等の溶媒、またはこれらの混合溶媒系、またはこれらにアセトン、アセトニトリルなどの極性溶媒を配合した混合溶媒系があげられる。ただしアルコール類は目的反応の基質となるため、基質として添加する以外に用いることはあまり好ましくない。

ホスホリパーゼDとしては、ホスファチジル基

転移活性を有するものであれば、市販のものあるいは公知の方法で調製したものを問わず使用できる。例として、ベーリンガー・マンハイム社（Boehringer Mannheim GmbH）製のキャベツ由来のホスホリパーゼD、東洋醸造㈱製の微生物由来のホスホリパーゼD（PLDP）、公知の方法（一例としてケーツとサストリイ(M.Kates and P.S. Sastry)の方法、"Methods in Enzymology" (J.M. Lowenstein, ed.), vol.14, pp197-203, Achademic Press, New York(1969)）により抽出し精製または部分精製した酵素標品、または抽出した粗酵素があげられる。

受容体としては、コリン、メタノール、エタノール、エタノールアミン、セリン、グリセロール、グルコース等の従来ホスファチジル基転移反応の受容体として知られている化合物のみならず、1-アミノ-2-プロパノール、1-オルソメチルグルコシド、トレハロースをはじめとする従来ホスファチジル基転移反応の受容体とはならないとされていた糖類を含む一級または二級アルコール構造を持つ化合物をも用いることができる。

ホスホリパーゼDを吸着あるいは担持させる担体の材質としては活性炭、活性白土、ケイ酸、シリカゲル、ケイ藻土、ゼオライト、アルミナ、多孔質ガラス、陶器、磁器、樹脂などがあげられ、形状としては粒径0.02～0.5mm 程度の粒状あるいはビーズ状が好ましい。またこれらの担体は、原料リン脂質および受容体を吸着あるいは担持させることもできる。

ホスホリパーゼDおよび受容体を担体に吸着あるいは担持させる方法の例としては、該成分の水溶液を担体と接触させ、ろ別後過剰の水を凍結乾燥し、あるいはジエチルエーテルまたはクロロホルム等の酵素が失活させない低級有機溶媒を繰り返しまたは連続的に接触させることにより除去する方法、あるいは粉末状にした該成分と担体とを混合したところへ微量の水を加えて更に混合する方法などがある。

酵素、受容体その他の成分を吸着または担持させた担体と、原料リン脂質を溶解または懸濁した有機溶媒とを接触させる方法としては、容器中で担体を溶媒系中に分散、懸濁し攪拌する方法、または担体をカラムなどに充填し溶媒系を循環させる方法などがある。

原料リン脂質を担体に吸着あるいは担持させる方法の例としては、低極性有機溶媒に溶解した原料リン脂質を担体と接触し吸着させた後、濾過により溶媒を除去する方法、あるいは原料リン脂質を溶解した有機溶媒を担体に含浸させた後、溶媒を溜去する方法などがある。

反応系全体の水分含量は、好ましくは1%以下に抑えることにより加水分解反応が従来の反応系に比べて抑制できるが、更に 0.5%以下に抑えることが好ましい。特に、水分含量を 0.2%以下に制御することにより従来の反応系では反応し得なかった受容体をも反応させることができ、本発明の効果が最大に発揮される。

反応温度は用いる酵素の至適温度であればよく、通常30～40℃の範囲である。ただし、用いる溶媒が低沸点のものである場合等はこの限りではない。

反応時間は 1.5～36時間で、好ましくは4～24時間である。

このようにして製造した任意の塩基を持つ目的リン脂質は溶剤分画、ケイ酸またはシリカゲルクロマトグラフィー、アルミナクロマトグラフィー、DEAE-セルロースクロマトグラフィー等の公知の手段を適宜用いることにより、容易に精製することができる。

（発明の効果）

本発明は、反応系中の水分含量を酵素が失活しない範囲で極限まで減少させるため、本質的に非水系の反応系を用いるので、従来の反応系で見られたようなPAの大量の生成は抑制され、反応後の目的リン脂質の分離精製が容易になり収率が向上した。

更に、従来の反応系では得ることのできなかった多くの種類の目的リン脂質をも製造することが可能となった。

（実施例）

以下、参考例、実施例、および比較例に基づい